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Genetics

Characterization and mutagenesis of the gene encoding the A49 subunit of RNA polymerase A in *Saccharomyces cerevisiae*

(interspecific complementation/ribosomal RNA)

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ABSTRACT **The gene encoding the 49-kDa subunit of RNA polymerase A in *Saccharomyces cerevisiae* has been identified by formation of a hybrid enzyme between the *S. cerevisiae* A49 subunit and *Saccharomyces douglasii* subunits based on a polymorphism existing between the subunits of RNA polymerase A in these two species. The sequence of the gene reveals a basic protein with an unusually high lysine content, which may account for the affinity for DNA shown by the subunit. No appreciable homology with any polymerase subunits, enzymes, or transcription factors is found. Complete deletion of the single-copy *RPA49* gene leads to viable but slowly growing colonies. Insertion of the *HIS3* gene halfway into the *RPA49* coding region results in synthesis of a truncated A49 subunit that is incorporated into the polymerase. The truncated and wild-type subunits compete equally for assembly in the heterozygous diploid, although the wild type is phenotypically dominant.**

The yeast *Saccharomyces cerevisiae* possesses three nuclear RNA polymerases—A, B, and C (I, II, and III). These three forms of RNA polymerase can be distinguished by their subcellular localization, chromatographic behavior, subunit composition, sensitivity to α-amanitin, and promoter/template specificity. Recent advances in the molecular genetics of yeast have made possible the functional dissection of the polymerases through cloning and sequencing of the genes encoding subunits and through selection of corresponding mutants (see refs. 1 and 2 for recent reviews).

The single (essential) function of RNA polymerase A (pol A) is to transcribe the >100 ribosomal DNA units into the 35S ribosomal precursor (3), which is subsequently matured into the functional 18S, 5.8S, and 25S species (4). These RNA species together comprise 85% of total cellular RNA (5). Like the two other nuclear enzymes, yeast pol A has a complex subunit structure that includes a core of four subunits (A190, A135, AC40, and AC19) related to the $\beta\beta'\alpha_2$ eubacterial core enzyme (6) and a set of five small subunits (ABC27, ABC23, ABC14.5, ABC10α, and ABC10β), which are common to all three yeast nuclear RNA polymerases (7). The corresponding genes have been cloned, sequenced, and found to encode essential components of the enzyme as judged from the properties of null mutants (6, 8–12). In addition, five other polypeptides (A49, A43, A34.5, A14, and A12.2) are associated with yeast pol A and are thus likely to be specific subunits of that enzyme (13, 14).

The gene encoding the 49-kDa subunit (A49) is of particular interest since this protein has been extensively studied at the biochemical level. Along with the subunit A34.5, A49 is easily dissociated from the rest of pol A, producing the form A*, which shows impaired transcriptional activity and increased sensitivity to α-amanitin as compared to the complete polymerase (14). In addition, the A49 subunit has been observed to copurify with an RNase H activity (15, 16), although the possibility of a contaminating enzyme with RNase H activity that copurifies with A49 has not yet been definitely excluded.

The present work describes the isolation and characterization of the gene encoding the A49 polypeptide.[‡]

MATERIALS AND METHODS

Yeast Strains and Genetic Techniques. The *S. cerevisiae* wild-type strain was CMY214 a/α *trp1-Δ1/trp1-Δ1 his3-Δ200/his3-Δ200 ura3-52/ura3-52 ade2-101/ade2-101 lys2-801/lys2-801 can1/CAN1* (7), and the *Saccharomyces douglasii* strain was 4707-22D aaa HO *his4 ura3 ade1 leu2* (from D. Hawthorne, University of Washington, Seattle). Yeast were grown on YPD (yeast extract/peptone/dextrose) (complete) medium or YNB (yeast nitrogen base) (minimal) medium appropriately supplemented. Cells were transformed by the lithium acetate technique (17), and standard genetic techniques for yeast (18) were used in analysis of transformants and meiotic progeny.

DNA. Cloning techniques were according to Maniatis *et al.* (19). DNA sequencing was carried out by subcloning *RPA49* fragments into M13 vectors followed by use of the Sanger dideoxynucleotide method (20) (using synthetic, specific oligonucleotide primers where appropriate). The vector pFL44 (21) is a 2-μm vector with *URA3* as a selective marker in yeast. The 1.7-kilobase (kb) *Bam*HI fragment used for gene disruption (22) includes portions of the two genes *DED1* and *PET56* (see Fig. 1). To construct the *rpa49-Δ::TRP1* allele, a 2.5-kb *Nru* I/*Spe* I fragment (Fig. 1) encompassing the wild-type *RPA49* gene was subcloned into a Bluescript vector, and an internal *Eco*RI/*Pst* I fragment was replaced by the *TRP1* gene derived from YRp7 (23), leaving 147 nucleotides of 5′ *RPA49* coding region. Both constructions were verified by Southern blotting.

Purification and Analysis of Pol A. Pol A was purified according to published procedures (24). For immunoblotting, samples were run on SDS/10% polyacrylamide gel and transferred to nitrocellulose. The filter was subsequently incubated with specific anti-yeast RNA polymerase antibodies at 15 μg/ml, washed three times with 10 mM Tris·HCl, pH 8/150 mM NaCl/0.5% Tween 20, and bound antibodies were immunoassayed using an alkaline phosphatase anti-rabbit IgG indicator reaction (Bio-Rad).

RESULTS

Cloning and Identification of the *RPA49* Gene. A DNA fragment potentially encoding part of the 49-kDa subunit of

Abbreviation: pol A, RNA polymerase A.
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[‡]The sequence reported in this paper has been deposited in the GenBank data base (accession no. M96600).

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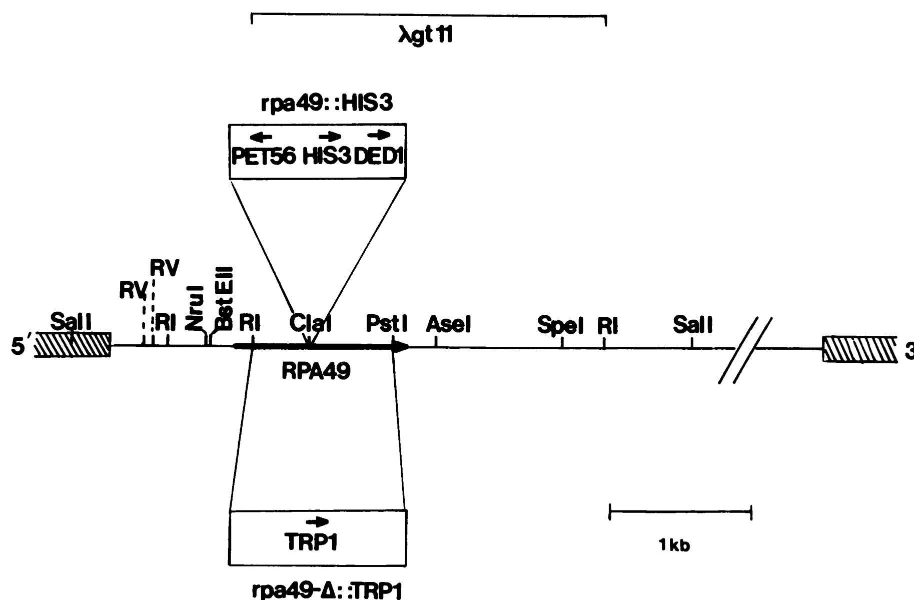

FIG. 1. Simplified restriction map showing relative positions of clones and gene disruptions. Region indicated in brackets corresponds to the original λgt11 insert, which produced a 190-kDa fusion protein with β-galactosidase. Hatched zone represents the vector YCp50, which carries a 10-kb fragment of yeast genomic DNA; thick arrow shows the *RPA49* coding region. *Eco*RI and *Eco*RV are shown as RI and RV, respectively. Mutants *rpa49*::*HIS3* and *rpa49*-Δ::*TRP1* are described in the text.

S. cerevisiae pol A was originally isolated as a fusion with *lacZ* by screening a λgt11 bank of yeast DNA with antibodies to the individual subunits of the three yeast RNA polymerases (25). We subsequently used a 0.45-kb *Cla* I/*Eco*RI subfragment to probe a yeast genomic DNA library in YCp50 to obtain the entire gene. In this way, we identified pYCp50-26, a plasmid that contained *RPA49* coding sequences at one end of a 10-kb yeast genomic insert. Northern blotting of poly(A) RNA extracted from CMY214, using a series of probes covering the *RPA49* region of the insert, showed a polyadenylylated transcript of the appropriate size for A49 (1.4 kb) and confirmed that the entire gene was present (data not shown).

To establish that the isolated gene encoded the *S. cerevisiae* A49 subunit, we were able to exploit a polymorphism of pol A existing between the two related yeast species *S. cerevisiae* and *S. douglasii* (24). Although the pol A enzymes of these two interfertile species have equal numbers of subunits, there are molecular weight polymorphisms for seven of the enzyme subunits. The *S. douglasii* subunit corresponding to A49 has an apparent molecular mass of 51 kDa by SDS/PAGE. Nevertheless, *S. douglasii* subunits are recognized by antibodies directed to *S. cerevisiae* pol A (24), implying a conservation of structure and, thus, function between the polymerase components of these two species.

A 3.8-kb *Sal* I fragment that spans the putative *RPA49* gene and includes 0.3 kb of YCp50 was cloned into the multicopy 2-μm vector pFL44 and introduced into *S. douglasii* strain 4707-22 by yeast transformation. Pol A preparations purified from the transformant and from a control strain were immunoblotted against *S. cerevisiae* A49-specific antibodies. This revealed the presence of two antigenic protein bands in the transformant at the positions corresponding to the *S. cerevisiae* and *S. douglasii* A49 and A51 subunits, respectively (Fig. 2*A*). Clearly, the *S. cerevisiae* subunit was expressed in the *S. douglasii* transformant. The *S. cerevisiae* A49 subunit cosedimented with the *S. douglasii* pol A in a glycerol gradient (Fig. 2*B*), indicating that the heterologous subunit was actually incorporated into pol A. However, the hybrid enzyme represented only ≈20% of the total *S. douglasii* pol A, based on the Western blot (Fig. 2*B*).

Sequence of the *RPA49* Gene. The *Eco*RV/*Ase* I interval encompassing *RPA49* (Fig. 1) was sequenced on both strands to reveal an open reading frame of 1248 nucleotides (Fig. 3), encoding a protein of M_r 46,633, a size slightly smaller than the size (M_r 49,000) derived from SDS/PAGE. The calculated codon bias index is 0.22, indicating a gene expressed at an average level (26). The high abundance of basic amino acid residues (44 lysines and 24 arginines) is consistent with the measured isoelectric point of 9 (27) and with the observation

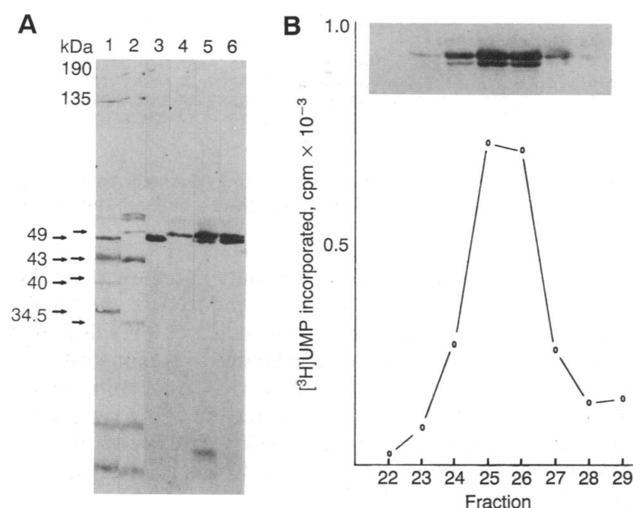

FIG. 2. (*A*) Immunodetection of the *S. cerevisiae* A49 subunit in a *S. douglasii* transformant. Purified pol A (4 μg) (glycerol gradient fractions) was immunoblotted against antibodies specific either to *S. cerevisiae* pol A (lanes 1 and 2) or to *S. cerevisiae* A49 subunit (lanes 3–6). Lanes: 1 and 3, *S. cerevisiae* wild type; 2 and 4, *S. douglasii* wild type; 5, *S. douglasii* transformed by the putative *S. cerevisiae RPA49* gene; 6, *in vitro* mixture of wild-type *S. cerevisiae* and *S. douglasii* polymerases. Vector control is not shown. (*B*) Cosedimentation of *S. cerevisiae* A49 subunit and *S. douglasii* pol A. Fractions from an SW65 10–30% (vol/vol) glycerol gradient were analyzed by immunoblotting as in *A*, using antibodies to A49 subunit. In parallel, 3-μl samples were assayed for pol A activity using poly[d(AT)] as template (14).

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-361  TTTAGCTTGTGGCGTTGGAACCTTCCATTTGGGCTGCACAATCTTCGGCG
-311  TAGACATTATAGTATATCTTCTCAGGGCTTTTATGAAGATATTCATGCAT
-261  AGAATAAGAACTTGACCGGACACCTAACACCGACGCCAATTAGCAATACT
-211  GTTCTTTTCAATTGATAATGTCTTTTGACTCTTCGCGATGAAAAATGTTT
-161  GCGGTCACCCGCGCTGAAAATTTTTTATTGCGATGAGCTGAAATTTTCCA
-111  TCTCATTTTTAATAGTAAGTATTCTTGCATGAACACTAATGATATTAGGA
 -61  GGGCTTTTTAAGTTGCTACCACTATTGCAGTCGTTATCAACCTTTTGCAC
 -11  TTTATCTAGTAATGTCCGTGAAAAGGTCTGTTTCTGAAATCGAAATTGAA
                    M  S  V  K  R  S  V  S  E  I  E  I  E
  39  AGTGTTCAAGATCAACCCTCTGTTGCCGTGGGAAGTTTCTTCAAGGGCTT
      S  V  Q  D  Q  P  S  V  A  V  G  S  F  F  K  G  F
  89  CCGTGCTCCCAGTGACACTACGTTCGACTTATACAAAAAAAAGAAGTCTG
       R  A  P  S  D  T  T  F  D  L  Y  K  K  K  K  S  E
 139  AAAAGGACGAATTCGTATTACACGGTGAAAACGAGAGACTAGAATACGAA
        K  D  E  F  V  L  H  G  E  N  E  R  L  E  Y  E
 189  GGTTATCCTGATTCTTCTTCCCAAGCTTCGAACCAGTATGTTGTGGGCCT
      G  Y  P  D  S  S  S  Q  A  S  N  Q  Y  V  V  G  L
 239  ATTTAATCCAGAAAAGAAAAGTATTCAACTCTACAAGGCTCCCGTACTTG
       F  N  P  E  K  K  S  I  Q  L  Y  K  A  P  V  L  V
 289  TTTCCAAAGTTGTATCCAAGTCAAGCAAGAATCTAAGGGGTCCAAAAATA
        S  K  V  V  S  K  S  S  K  N  L  R  G  P  K  I
 339  AAAAGTAAGAGTGATACTCGTCCATCTGCTTTGAGAAATGCGTTGGGTGA
      K  S  K  S  D  T  R  P  S  A  L  R  N  A  L  G  E
 389  AGCGTTTGGTACTAAAAAGGCTAAAAAAGCTATTGCAGATCTAGAAAGAA
       A  F  G  T  K  K  A  K  K  A  I  A  D  L  E  R  N
 439  ACCGTATTGACTCTGATAAGTTGACTGATTGTGCTATTGACATTGTGGAT
        R  I  D  S  D  K  L  T  D  C  A  I  D  I  V  D
 489  TCCGTGAGAACTGCATCTAAGGATTTACCAACTCGGGCTCAATTGGATGA
      S  V  R  T  A  S  K  D  L  P  T  R  A  Q  L  D  E
 539  AATTACTTCCAACGATAGACCTACTCCATTAGCCAATATCGATGCCACTG
       I  T  S  N  D  R  P  T  P  L  A  N  I  D  A  T  D
 589  ATGTAGAACAAATTTACCCAATTGAAAGTATAATACCAAAGAAGGAATTA
        V  E  Q  I  Y  P  I  E  S  I  I  P  K  K  E  L
 639  CAGTTTATTCGCGTTTCATCAATTCTGAAAGAAGCGGATAAGGAAAAGAA
      Q  F  I  R  V  S  S  I  L  K  E  A  D  K  E  K  K
 689  GTTGGAATTGTTCCCATACCAAAACAATTCCAAGTATGTGGCAAAGAAAT
       L  E  L  F  P  Y  Q  N  N  S  K  Y  V  A  K  K  L
 739  TGGACTCTTTGACACAACCTTCACAAATGACCAAATTACAGCTACTATAC
        D  S  L  T  Q  P  S  Q  M  T  K  L  Q  L  L  Y
 789  TACTTATCACTATTACTGGGCGTATACGAAAATAGACGCGTGAATAACAA
      Y  L  S  L  L  L  G  V  Y  E  N  R  R  V  N  N  K
 839  GACGAAGTTGTTAGAAAGGTTGAACAGTCCTCCTGAAATCTTGGTAGATG
       T  K  L  L  E  R  L  N  S  P  P  E  I  L  V  D  G
 889  GTATCTTAAGCAGATTTACCGTGATAAAGCCTGGACAGTTTGGAAGATCA
        I  L  S  R  F  T  V  I  K  P  G  Q  F  G  R  S
 939  AAGGATCGCTCCTATTTCATTGACCCACAAAATGAAGATAAAATCTTATG
      K  D  R  S  Y  F  I  D  P  Q  N  E  D  K  I  L  C
 989  CTACATCTTGGCAATCATAATGCATTTGGATAACTTCATTGTTGAAATCA
       Y  I  L  A  I  I  M  H  L  D  N  F  I  V  E  I  T
1039  CACCCTTAGCACACGAATTGAACTTGAAACCTTCCAAAGTTGTCAGTTTG
        P  L  A  H  E  L  N  L  K  P  S  K  V  V  S  L
1089  TTCAGGGTACTTGGTGCTATTGTCAAAGGTGCCACAGTGGCTCAAGCTGA
      F  R  V  L  G  A  I  V  K  G  A  T  V  A  Q  A  E
1139  GGCTTTTGGCATTCCAAAAAGTACTGCAGCTTCTTATAAAATTGCCACTA
       A  F  G  I  P  K  S  T  A  A  S  Y  K  I  A  T  M
1189  TGAAAGTTCCATTTAAGCTACCTGAAATGACAAGAAGAGGAAGAGGTCCA
        K  V  P  F  K  L  P  E  M  T  R  R  G  R  G  P
1239  AGACGTTAGAGTTACCTAGAGGTATATCTGAGCATTTGGTTTGCTCTGGT
      R  R  *
1289  TTTTTTTTTTGCTACATGGTTCTATAAAGCAGCAAAATATAAGACGGTCC
1339  TCCTGATGAGAGGAAGAGAAAAACAAAAATAACTACATAGATATACGCTA
1389  TTTTTTCTGAGAAGATACATATGTACAAATAGATATTTTACAATAATTTT
1439  AAACTTCGCAATAAATTCAATATTAATTCATAGCGTACTTCGGCTTTCTA
1489  GCACCTTAGTAAGCTGAAATTTAAAAA
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FIG. 3. Nucleotide sequence of the *RPA49* gene. Amino acid sequence predicted for the A49 protein is shown beneath the coding region.

that the A49 protein binds to DNA (data not shown). In the context of DNA binding, it is interesting to note the highly basic C terminus of the protein, at which 5 of 9 residues are arginines. However, we have been unable to demonstrate the formation of sequence-specific A49–rRNA-encoding DNA complexes. The *RPA49* sequence does not show significant homology with any subunits from yeast or *Escherichia coli* RNA or DNA polymerases or with RNase H enzymes or transcription factors.

Disruption of the *RPA49* Gene. Southern blot experiments demonstrated that the *RPA49* gene was present in a single copy per haploid genome (data not shown). Two mutant alleles of *RPA49* were made by homologous recombination using the one-step gene disruption technique (28). To construct the *rpa49::HIS3* allele, a *Bam*HI fragment carrying *HIS3* was inserted into the RPA49 *Cla* I site situated about halfway into the coding region in YCp50-26 (Fig. 1), and the plasmid was digested with *Eco*RI to target specific integration at the chromosomal *RPA49* locus. In the case of the null allele *rpa49*-Δ::*TRP1*, digestion of the plasmid with *Bst*EII and *Spe* I prior to transformation directed replacement of most of the

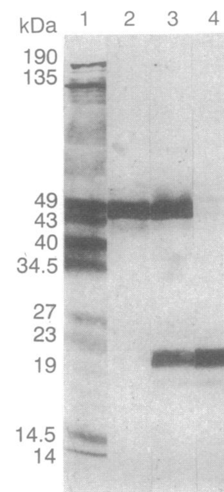

FIG. 4. Composition of pol A in *rpa49::HIS3* disrupted strains. Samples of glycerol gradient-purified polymerase (5 μg) were immunoblotted against antibodies specific to pol A (lane 1) or to A49 subunit (lanes 2–4). Lanes: 1 and 2, control wild type (diploid); 3, diploid heterozygous for the *rpa49::HIS3* disruption; 4, *rpa49::HIS3* haploid.

RPA49 gene by *TRP1* sequences. Use of the diploid strain CMY214 *CAN1/can1* as the host for integrative transformation made it possible to carry out analysis of spores either by tetrad dissection or by selection of random spores for the recessive canavanine-resistant phenotype on appropriately supplemented media. Tetrad analysis of the complete gene disruption showed that *rpa49*-Δ::*TRP1* invariably cosegregated with slow growth in a Mendelian (2:2) fashion. Furthermore, using random spore analysis, tiny *rpa49::HIS3* colonies carrying the partial gene disruption were recovered on YPD. The two mutants were phenotypically indistinguishable, each having a doubling time 2–3 times that of wild type. In both cases, the *RPA49* plasmid pFL44-49 was able to restore normal growth to the small colonies, confirming that the phenotypes were due to mutation of the *RPA49* gene. The ability of the clones to grow on glycerol excluded the possibility of a secondary mitochondrial mutation giving a petite phenotype. Interestingly, in the case of the *rpa49::HIS3* mutant but not in that of the deletion mutant, suppression events occurred after several generations in liquid culture, allowing the cells to regain their normal growth rate. The mechanism of this suppression was not investigated.

Immunoblotting of pol A from the disrupted strains showed that in the mutant *rpa49::HIS3*, a 23-kDa polypeptide cross-reacting with anti-A49 antibodies has replaced the wild-type A49 subunit (Fig. 4). Based on the sequence of the *HIS3* insertion in *RPA49*, one can predict the presence of two possible fusion proteins. These would be a 21.5-kDa polypeptide under the control of the *RPA49* promoter and a 25.1-kDa *DED1–RPA49* fusion protein (see Fig. 1). Therefore, one could not conclude whether the truncated subunit derived from the N-terminal or the C-terminal part of A49. In the case of the null allele *rpa49*-Δ::*TRP1*, as expected, partially purified pol A did not contain any polypeptide cross-reacting with anti-A49 antibodies (data not shown).

The pol A purified from the diploid heterozygous for the disruption can be seen to incorporate both the wild-type and truncated subunits in comparable amounts (Fig. 4). However, although the two types of subunit appear to compete equally for assembly into the polymerase, the reduced growth of the haploid-disrupted strain indicates that the truncated polypeptide is functionally inadequate. The wild-type growth of the heterozygous diploid furthermore shows that the wild-type allele is dominant (data not shown).

DISCUSSION

We were able to exploit a polymorphism of pol A subunits existing between the two related yeast species *S. cerevisiae* and *S. douglasii* (24) to identify the gene encoding A49. Assembly of the *S. cerevisiae* A49 subunit into *S. douglasii* pol A clearly showed that *RPA49* had been cloned. This was further substantiated by the molecular weight and isoelectric point of the protein predicted from the nucleotide sequence, which correlated well with previously established properties of the A49 protein (27). The fact that $<20\%$ of the *S. douglasii* pol A incorporates the heterologous subunit, although the *S. cerevisiae* subunit is on a multicopy plasmid, could be due to poor expression of the gene in the heterologous environment, inefficient assembly of the heterologous subunit, or preferential degradation of the *S. cerevisiae* subunit as shown to be the fate of excess RNA polymerase β and β' subunits in *E. coli* mutants (29). Polymorphism has been shown to occur among the ribosomal proteins of 17 yeast species of the genera *Saccharomyces* and *Kluyveromyces* (30) and is also likely the case among other essential and hence potentially highly conserved multicomponent elements of the cellular machinery. The approach we have used to identify *RPA49* should therefore be useful for cloning other yeast genes encoding polymorphic proteins.

We have shown that both complete and partial deletion mutations of the single chromosomal *RPA49* gene permit yeast growth, indicating that the A49 subunit is not strictly required. We have taken advantage of the viability of the two disrupted alleles to examine the subunit structure of the mutant pol A by immunoblotting. Unlike the situation observed *in vitro* (14), the absence of A49 from the polymerase was not accompanied by the dissociation of A34.5 (data not shown). Although the reduced growth rate of the haploid *rpa49::HIS3* mutant shows that pol A possessing the truncated form of A49 is defective, in the diploid heterozygote, equal amounts of the truncated and wild-type A49 subunit are incorporated into pol A. In other words, there is neither degradation of the defective enzyme or truncated subunit nor increased synthesis of the wild-type subunit. Thus, it can be concluded that the truncated polypeptide competes efficiently with the wild-type subunit for assembly into the enzyme, although the normal growth of the heterozygote shows that the wild-type allele is dominant.

In RNA labeling experiments, the rate of synthesis of 5.8S rRNA (processed from the 35S rRNA transcript) versus 5S RNA (an RNA polymerase C transcript) was found to be markedly reduced in the *rpa49-Δ::TRP1* mutants (S. Stettler and P. Thuriaux, personal communication), showing that, despite the dispensable nature of the A49 subunit, it is important in rRNA synthesis. The observation that the A* form of pol A transcribes poly[d(AT)] as efficiently as the intact polymerase at low-salt concentration (14) implies that A49 is not essential for chain elongation. The precise function of the A49 subunit in rRNA metabolism thus remains unknown, although it does not appear to be a critical element in polymerase assembly or in RNA chain elongation. The function of A49 might be linked to the intriguing RNase H activity that was found associated with this subunit (15, 16). It is also interesting to note that A49 can be found in a high-salt chromatin extract, dissociated from enzyme A (16), which suggests that it might also possess some role in chromatin extending beyond RNA polymerase function.

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